# Molecular switches facilitate rhythms in the circadian clock of *Neurospora crassa*

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# *Abstract*

Lebenswissenschaftlichen Fakultät
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Doctor rerum naturalium

**Molecular switches facilitate rhythms in the circadian clock of *Neurospora crassa***

by Abhishek, UPADHYAY

Chronobiology is the study of periodically occurring physiological, metabolic, and behavioral processes in living organisms. Circadian rhythms have evolved across cyanobacteria, algae, fungi, insects, plants and mammals based on daily interactions between internal timing and environmental cues. Circadian oscillators consist of a transcription-translation feedback loop (TTFL) allowing self-sustained rhythms. A delayed negative feedback loop is central to this gene regulatory network.

Theory predicts that self-sustained oscillations require robust delays and nonlinearities (ultrasensitivity). We study the circadian rhythms in the filamentous fungi *Neurospora crassa* (wild type period 22.5 hours) to investigate the underlying clock mechanisms. Its transcription translation feedback loop (TTFL) includes the activator White Collar Complex (WCC) (heterodimer of WC1 and WC2) and the inhibitory FFC complex, which is made of FRQ (Frequency protein), FRH (Frequency interacting RNA Helicase) and CK1a (Casein kinase 1a). Moreover, there are multiple phosphorylation sites on FRQ (around 100) and WCC (approximately 95). FRQ is phosphorylated by CK1a in a sequential distributive manner. While exploring the temporal dynamics of these proteins, we investigate:

1) how multiple, slow and random phosphorylations govern delay and nonlinearity in the negative feedback loop.

2) how limit cycle oscillations arise and how molecular switches support self-sustained rhythms.

In the first publication contained in this thesis, we simulate FRQ multisite phosphorylations using ordinary differential equations. The model shows temporal and steady state switches for the free kinase and the phosphorylated protein. The model can further be utilized to study the priming-dependent and independent phosphorylations by CK1a.

In the second publication presented, we developed a mathematical model of 10 variables with 26 parameters to understand the interactions and feedbacks among WC1 and FFC elements in nuclear and cytoplasmic compartments. Our control and bifurcation analysis showed that the model produces robust oscillations. Our model revealed a switch between WC1-induced transcription and FFC-assisted inactivation of WC1. Using this model, we also studied possible mechanisms of glucose compensation. This model can further be utilized to study entrainment and temperature compensation.

In summary, the core clock of *Neurospora* was examined and mechanisms underlying molecular switches were revealed.

HUMBOLDT-UNIVERSITÄT ZU BERLIN

# *Zusammenfassung*

Lebenswissenschaftlichen Fakultät
Institut für Theoretische Biologie

Doctor rerum naturalium

**Molekulare Schalter erleichtern Rhythmen in der zirkadianen Uhr der Neurospora crassa**

von Abhishek, UPADHYAY

Chronobiologie ist das Studium der periodisch auftretenden physiologischen, metabolischen und verhaltensbezogenen Prozesse in lebenden Organismen. Zirkadiane Rhythmen haben sich bei Cyanobakterien, Algen, Pilzen, Insekten, Pflanzen und Säugetieren auf der Grundlage täglicher Wechselwirkungen zwischen internem Timing und Umweltreizen entwickelt. Zirkadiane Oszillatoren bestehen aus einer Transkriptions-Translations-Rückkopplungsschleife (TTFL), die selbsterregte Rhythmen ermöglicht. Eine verzögerte negative Rückkopplungsschleife ist zentral für dieses genregulatorische Netzwerk.

Die Theorie sagt voraus, dass selbsterregte Oszillationen robuste Verzögerungen und Nichtlinearitäten (Ultrasensitivität) erfordern. Wir untersuchen die zirkadianen Rhythmen in dem filamentösen Pilz *Neurospora crassa* (Wildtyp-Periode 22,5 Stunden), um die zugrundeliegenden Uhrmechanismen zu studieren. Seine Transkriptions-Translations-Rückkopplungsschleife (TTFL) umfasst den aktivierenden White Collar Complex (WCC) (Heterodimer aus WC1 und WC2) und den hemmenden FFC-Komplex, der aus FRQ (Frequency protein), FRH (Frequency interacting RNA Helicase) und CK1a (Caseinkinase 1a) besteht. Darüber hinaus gibt es mehrere Phosphorylierungsstellen auf FRQ (etwa 100) und WCC (etwa 95). FRQ wird von CK1a in einer sequentiellen, distributiven Weise phosphoryliert. Während wir die zeitliche Dynamik dieser Proteine erforschen, untersuchen wir: 1) wie multiple, langsame und zufällige Phosphorylierungen die Verzögerung und Nichtlinearität in der negativen Rückkopplungsschleife bestimmen. 2) wie Grenzzyklus-Oszillationen entstehen und wie molekulare Schalter selbsterregte Rhythmen unterstützen.

In der ersten Veröffentlichung dieser Arbeit simulieren wir FRQ-Multisite- Phosphorylierungen mit Hilfe gewöhnlicher Differentialgleichungen und probabilistische Methoden. Das Modell zeigt zeitliche und stationäre Schalter für die freie Kinase und das phosphorylierte Protein. Das Modell kann ferner zur Untersuchung der Priming-abhängigen und -unabhängigen Phosphorylierungen durch CK1a genutzt werden.

In der zweiten vorgestellten Publikation entwickelten wir ein mathematisches Modell von 10 Variablen mit 26 Parametern, um die Interaktionen und Rückkopplungen zwischen WC1- und FFC-Elementen in Kern- und Zytoplasma- Kompartimenten zu verstehen. Unsere Kontroll- und Bifurkationsanalyse zeigte, dass das Modell robuste Oszillationen erzeugt. Unser Modell offenbarte einen Wechsel zwischen WC1-induzierter Transkription und FFC-unterstützter Inaktivierung von WC1. Mit diesem Modell haben wir auch mögliche Mechanismen der Glukosekompensation untersucht. Unser Modell kann weiter zur Untersuchung von Entrainment und Temperaturkompensation verwendet werden.

Zusammenfassend wurde die Kernuhr von *Neurospora* untersucht und dabei die Mechanismen, die den molekularen Schaltern zugrunde liegen, aufgedeckt.

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# Declaration of Authorship

I, Abhishek, UPADHYAY, declare that this thesis titled, "Molecular switches facilitate rhythms in the circadian clock of *Neurospora crassa*" and the work presented in it are my own. I confirm that:

- This work was done wholly while in candidature for a research degree at this University.
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.

Signed: _____

Date: _____

# Contents

# Chapter 1

# Introduction

## 1.1 Biological Background

While a separate introduction is given in sections 2.1.1 and 2.2.1 for each part of the work presented in this thesis, this section contains a brief overview combining aspects of both parts.

### 1.1.1 Circadian rhythms in *Neurospora crassa* and role of molecular switches in the clockwork

The Earth's rotation around its own axis produces the 24-h day and night cycles. Life on earth in forms of cyanobacteria, algae, fungi, plants and animals has evolved 24 hour periodicities called circadian clocks [1, 2, 3]. This time-keeping molecular machinery helps to anticipate daily environmental signals such as light, temperature and nutrients. Circadian rhythms regulate a wide variety of molecular and physiological processes [4, 5, 6].

Circadian oscillators are based on a transcription-translation feedback loops. A delayed negative feedback loop is central to the gene regulatory network [7]. The core negative feedback loop of the fungal clock contains the negative element FREQUENCY (FRQ), which inhibits its own expression via inhibition of the circadian transcription factor White Collar Complex (WCC). FRQ is an intrinsically disordered protein (IDP) progressively hyperphosphorylated mainly by CK1a (Casein Kinase 1a). Hyperphosphorylation ultimately leads to functional inactivation and degradation of FRQ. It further allows the WCC to resume a new cycle [8, 9, 10].

The core clock gene network of the negative feedback loop has been considered for rhythm generation in this study. However, in principle several feedback loops exist that could contribute to rhythm generation in *Neurospora*.

A molecular switch is a biomolecule that can be reversibly or irreversibly changed between two or more stable states. The molecules may be shifted between the states in response to environmental cues, such as light, temperature, nutrients, changes in pH, or in the presence of ions and other ligands. Molecular switches are part and parcel of the biochemical processes. Many biological functions are regulated by molecular switches, for example allosteric, photochromic etc. [11]. Furthermore, in circadian biology, molecular switches seem to play an important role.

A delayed negative feedback loop in circadian rhythms includes several processes like epigenetic changes, transcription, translation, nuclear transport, post-transcription and post-translation modifications, mRNA decay, and proteasomal degradation [12, 13, 14]. There are potential candidates for molecular switches across these processes. Recent experiments suggest that many sites on FRQ (about 100 sites) are phosphorylated over the course of many hours at a seemingly random

manner. Moreover the activator WCC (about 95 sites) also gets phosphorylated [15, 16, 17]. Phosphorylations govern nuclear translocation, complex formations, inactivation of transcription and stability and significantly contribute to the required delay [18, 19, 20]. Therefore understanding the mechanism of multiple random phosphorylation in the generation of switch-like behaviour and delay in clockwork is of general interest.

### 1.1.2 Motivation and project outline

Understanding circadian clocks quantitatively helps to explore their functional significance in different organisms. In mammals, a functional clock can help to prevent diseases such as cancer, obesity, and depression [4, 5, 21]. To reveal the basic mechanisms of the underlying gene-regulatory network, *Neurospora crassa* has been established as a useful model organism. A Transcriptional-Translational Feedback Loop (TTFL) serves as the generator of self-sustained oscillations with a period of about a day [8], but quantitative details of these delayed negative feedback and supporting molecular switches are not well understood. For example, the inhibition of WCC via FFC might involve hyperphosphorylation, sequestration, and stoichiometric inhibition [18, 22, 23, 24]. Moreover, it is not clear how the long delay is realized to obtain daily rhythms.

Thus, this gives rise to two general questions: (1) How can multiple random phosphorylations produce switch-like behaviour and long delays? (2) What are the underlying switch mechanisms of negative feedbacks? The focus of our study is a deeper understanding of the molecular switches involved in the *Neurospora* clock.

Recent experiments suggest that slow and seemingly random phosphorylations of intrinsically disordered clock protein FRQ control stability and function of clock protein complex FFC in *Neurospora* [15, 25]. Oscillator theory predicts that self-sustained circadian rhythms ("limit cycles") require long delays and nonlinearities such as switches [26, 27]. To understand the role of multiple random phosphorylation in clocks, we designed an in silico study in which we developed ordinary differential equation models. These conceptual mathematical models reflect the complexity of multiple random phosphorylations. We systematically compared several generic models describing linear processive phosphorylation, nonlinear distributive phosphorylation, and random phosphorylation [28]. Interestingly, we found that long delays are robustly achieved by extended reaction chains and slow degradation. Sequestration enhances the formation of temporal switches with high Hill coefficients. Thus our simulations supported the hypothesis that multiple random phosphorylation can provide robust delayed switches in clocks [28].

Given the insufficient detailed knowledge of the negative feedback in *Neurospora* clock, a second question addresses a possible mechanism for rhythms generation. To this end, we developed a mathematical model to explore the interactions and feedback among WC1 and FFC elements in nuclear and cytoplasmic compartments of *Neurospora* clock. We identified an ON/OFF switch of the frequency gene as a sharp transition between active transcription with slow turnover of WC1 and fast FFC-assisted inactivation of WC1. We performed control and bifurcation analysis to show that our novel model produced robust oscillations and reproduced basic features of wild-type rhythms and selected mutants. A comparison with the literature is consistent with the mutations studies and knockout experiments. Using the new model, we also study possible mechanisms of glucose compensation. A fairly simple

model with just three nonlinearities elucidated temporal clock dynamics, revealing a mechanism of rhythms'production [20].

## 1.2 Theoretical Background

The methods used in this thesis are in detail described in the respective publications. Nevertheless, I wish to give an overview over two basic concepts that are central for the presented work.

### 1.2.1 Modelling circadian clocks using differential equations

**Ordinary differential equations** Mathematical models in theoretical chronobiology are predominantly based on ordinary differential equations (ODEs) [29]. A change of concentration of substances over time is described using ODEs. For example, a substance x represents the change of its concentration over time with a proportional reaction rate. Here, k is a rate constant. This type of association is also called mass action kinetics [30].

$$\frac{dx}{dt} = k \cdot x \tag{1.1}$$

More generally ODEs can have the form

$$\frac{dx}{dt} = f(x,t) \tag{1.2}$$

where the function f may represent different types of kinetics, which may also be nonlinear. Besides mass action kinetics, Michaelis-Menten kinetics are frequently used [31]. Hill kinetics can be described as:

$$f(x,t) = \frac{x^h}{K^h + x^h} \tag{1.3}$$

For x much smaller than the constant K the term is close to 0, while for x much larger than K it it close to 1 and when x = K, and h = 1, it is 0.5. The constant h is called Hill coefficient. It describes the degree of nonlinearity. Thus it governs how fast f (x, t) switches from 0 to 1 for increasing x.

The solutions of ODEs depend on the initial conditions. In numerical simulations, the changes described by the ODEs are derived from initial values. Therefore, different initial conditions lead to different trajectories [30].

**Generation of oscillations** An ODE system (a system of multiple ODEs) represents several substances that regulate each others concentrations. The dynamics of the system is governed by an ODE system. Here is a simplified example of the Goodwin oscillator [32]:

$$\frac{dx}{dt} = \frac{1}{1+z^h} - k_1 \cdot x \tag{1.4}$$

$$\frac{dy}{dt} = x - k_2 \cdot y \tag{1.5}$$

$$\frac{dz}{dt} = y - k_3 \cdot z \tag{1.6}$$

For example, x, y and z might represent mRNA, cytosolic and nuclear protein concentrations of a gene, respectively. Each equation has a production (positive) and a degradation term (negative). Note, that there is a nonlinear production term in equation (1.4), which could represent inhibition of the mRNA concentration by the nuclear protein. Constant values for h, $k_1$ , $k_2$ and $k_3$ are called parameters. The system may exhibit different behaviour for different values of the parameters. Note, that the model described in equations (1.4) to (1.6) could be sufficient to generate rhythms, if the Hill-coefficient is large enough.

Note also, that the three equations (1.4, 1.5, and 1.6) form a negative feedback loop, since x activates y (x $\rightarrow$ y), y activates z (y $\rightarrow$ z) and z inhibits x (z $\dashv$ x). Note, that a large nonlinearity such as a high Hill-coefficient is not sufficient for rhythm generation, therefore, a negative feedback is essential [33]. A delay is also needed in this feedback loop. Here, the delay is implicitly included via a chain of x, y and z. Otherwise, a delay can explicitly be included by using delay differential equations.

### 1.2.2 Dynamical systems and control analysis

**Time series** A time series is essentially a set of data points ordered in time. In a time series, time is usually the independent variable. In the example above, data points for the variables x, y, and z can be plotted against variable t. An attractor is a set of numerical values towards which a dynamical system (e.g. Goodwin oscillator) tends to evolve, for a diverse range of initial conditions. A trajectory of the system in the attractor should remain on the attractor, onward in time. The trajectory may be periodic or chaotic. A stable limit cycle is a closed trajectory in phase space having the property that other trajectories spiral into it as time approaches infinity (t $\rightarrow$ $\infty$)[30].

**Bifurcation diagrams** A bifurcation diagram shows qualitative changes, for example the start of rhythms, as a function of a bifurcation parameter. A Hopf bifurcation occurs when a transition from damped to self-sustained rhythms takes place [20]. Moreover, two attractors - the limit cycle and steady state - may coexist while the parameter is slowly varied. Such a phenomenon is called as co-existence of attractors [34].

**Sensitivity analysis** Sensitivity analysis is the study of how the uncertainty in the output of a system can be allocated to its inputs. In our work, we are interested in the changes in the output of clock model system, i.e., period ($\triangle T$ =T$_{perturbed}$ - $T_{unperturbed}$) due to changes in inputs, i.e., kinetic parameters ($\triangle par$ =par$_{perturbed}$ - $par_{unperturbed}$). To quantify period changes for varying parameters, we can calculate the control coefficients ($C_{per}$) using equation 1.7 by quantifying the sensitivity of the system [35]. A parameter can be changed by +/- 10 percent, and the corresponding period change can be extracted from the simulations [35].

$$C_{per} = \frac{\triangle T / T_{unperturbed}}{\triangle par / par_{unperturbed}} = \frac{\triangle T / T}{0.1} \tag{1.7}$$

Note, that a positive period control coefficient of 0.5 would imply that a 10 percent parameter increase induced a circadian period lengthening by five percent, i.e., about 1 hour.

### 1.2.3 Switches

**Bistability** Dynamical systems describe oscillations and bistability in biological systems. Bistability means that the system may have two stable steady states. Bistability can be characterized by low and high steady state values [33, 36]. There are mainly two types of bistability: reversible and irreversible. Reversibility is induced by a transition from bistability to monostability and vice versa over increasing or decreasing input value. In bistability with hysteresis, there are two stable steady states output for each input value. However, an irreversibility is generated by a one-way switch with a transition from bistability to monostability and vice versa over increasing or decreasing input value [33].

**Ultrasensitivity** Ultrasensitivity is an attribute of a steady state input-output relationships that moulds them switch-like in character. An ultrasensitive response is often sigmoidal and the switching curve can be well approximated by the Hill equation [36, 37, 38]. The effective Hill equation is defined for the curves (see Equation 1.8). The effective Hill coefficient h is related to the effective concentration 90% (EC90) and 10% (EC10) ratio by Equation 1.9. Here K is the effective concentration when 50% (or EC50) of total signal is achieved.

$$y = \frac{x^h}{K^h + x^h} \tag{1.8}$$

$$h = \frac{log[81]}{log[EC90/EC10]} \tag{1.9}$$

In summary, an input–output relationships is ultrasensitive if it takes less than an 81-fold change in input to drive the output from 10% to 90% of maximum [37].

# Chapter 2

# Publications

## 2.1 Published work 1: a delayed negative feedback is core of the circadian clock

### 2.1.1 Context

**The fundamental mechanism:** The nobel prize 2017 in physiology or medicine was awarded for the discovery of a first clock protein, PER, in the fruit fly [39]. Since the discovery in 1984, an increasing number of clock components has been identified. The homologs of PER and additional proteins in circadian clocks of mammals and fungi have also been identified [40, 41].

Around the same time in 1986, the FREQUENCY (FRQ) protein was identified as the core clock protein that is responsible for circadian rhythms in *Neurospora crassa*. In 1990, a transcriptional-translational negative feedback loop (TTFL) was proposed as the key mechanism for rhythm generation [7]. It was conceptualized that long delays and nonlinearities ("switches") are required for self-sustained rhythms (“limit cycles”). The core delayed negative feedback loop of the fungal circadian clock is essentially closed with an autoinhibition of FRQ expression via inhibition of the transcription factor White Collar Complex (WCC) [9, 42, 43].

**Sources of required delays and switch-like inhibition:** Diverse processes have been associated to *Neurospora* clock function. For example, chromatin remodelling via the binding of clock components, transcription, splicing, mRNA decay, nuclear import and export, translation, complex formation, post-translational modifications and proteasomal degradation [15]. Importantly, these processes add to the overall delay required for the clock to function. However, according to mathematical theory 6-8 hours of delay is needed in periodic oscillation of 24 hours [44, 45]. The half-life of the FRQ is only about 3-5 hours [46]. We, therefore, searched for one of the key processes which may significantly contribute to long delays.

Interestingly, FRQ, an intrinsically disordered protein (IDP), makes a stable complex with FRQ-INTERACTING HELICASE (FRH) and is mainly hyperphosphorylated by casein kinase 1a (CK1a) in a seemingly random manner. In addition, the FFC complex interacts with WCC. Hyperphosphorylation of FRQ eventually leads to functional inactivation and degradation of FRQ. Finally, disassembly of the FFC complex allows the WCC to resume a new cycle [8, 9, 10]. Therefore, multiple random phosphorylations seem to be implicated in providing long delays and switch-like behaviour.

**Unresolved role of multiple random phosphorylation:** Of late about 100 phosphorylation sites in FRQ have been identified [15]. Phosphorylations of those sites might regulate cellular processes in various ways. There is a positively charged

N-terminal part and a negatively charged C-terminal part of FRQ protein. Initial phosphorylations seem to stabilize a closed conformation whereas progressive hyperphosphorylations supports an open conformation potentially via charge repulsion [15, 47]. Thus, we conceived that the stability may be governed by the overall number of phosphorylated sites.

While the progressive hyperphosphorylation of FRQ correlates with circadian timekeeping, the functions of phosphorylation are not understood in detail [15]. Moreover there is no understanding of how time is mechanistically measured by phosphorylation. We hypothesized that multiple phosphorylation could provide a mechanism to achieve long delays and switch-like inactivation of the FFC.

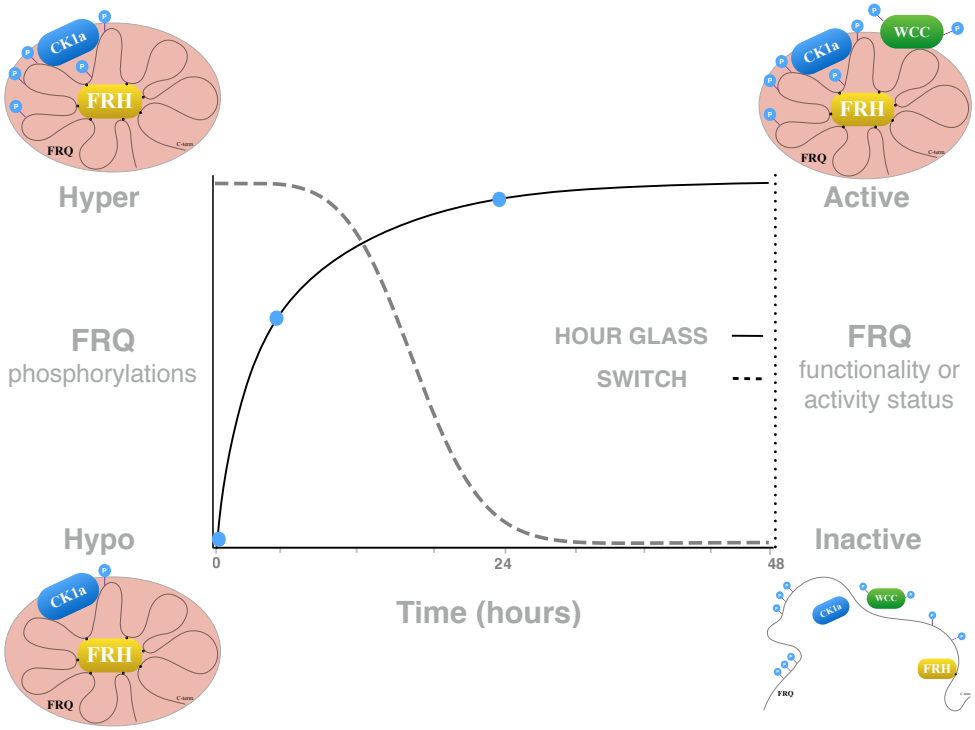


FIGURE 2.1: An hourglass and an activity switch based on multiple phosphorylations. Adapted from [28]: A delayed negatived feedback of the progressive phosphorylations of FRQ and a phosphoswitch in the *Neurospora* clock.

### 2.1.2 Research question and findings

**Research question:** Given the complexity of the clock protein interactions shown in Figure 2.1 and the delayed switch mechanism contained in this network, we asked how the delays and switch-like behaviour, most essential for clock, were generated.

There are experimental evidences for a slow and random phosphorylation of the clock phosphoprotein FRQ [19, 48]. Fig 2.1 reflects progressive phosphorylation in *Neurospora*. FRQ is stabilized by the FRH and forms the FFC complex with the kinase CK1a. FRQ undergoes slow, seemingly random multiple phosphorylations. At a hypercritical phosphorylation level the complex gets inactivated in a switch-like manner [47, 49]. We were thus interested, how multiple random phosphorylation

provide long delays and switches needed for negative feedback in circadian clocks.

**Approach:** Using conceptual models of sequential and distributive phosphorylations, we systematically explored in generic models how multiple phosphorylations could produce long robust delays and switch-like behaviour [28].

Recent experiments show that in *Neurospora* about 100 FRQ sites are phosphorylated over more than a circadian day (up to 48 hours) in a seemingly random manner [15, 16]. We designed conceptual models of multiple phosphorylations. We, firstly, simulated 4 phosphorylation sites using linear differential equations for processive phosphorylation where the kinase maintains a continuous binding to the substrate [28, 50]. For simplicity, we did not explicitly consider phosphatases.

Furthmore, dissociation of kinase and rebinding to other FRQ molecules could be represented as a distributive mechanism [50]. Interestingly, an ultrasensitive responses in protein phosphorylations may be generated via distributive enzyme kinetics [36, 51]. Therefore we developed nonlinear differential equations describing distributive phosphorylation.

There is some evidence that FRQ is phosphorylated by CK1a in a seemingly opportunistic manner [15, 25]. Conceivably, the highly flexible FRQ protein may randomly interact with the active site of CK1a. It may, in turn, lead to the initiation of phosphorylation [52]. Therefore we simulated random phosphorylations. We introduced new variables F1, F2, etc. so that simulations with differential equations would stay feasible. Hence, all F molecules with 1, 2, ... phosphorylations could be combined using these variables. That is to say, Fk constitutes the $\binom{n}{k}$ molecules with k out of n phosphorylations. Notably, our nonlinear random model contained in total only 2n+2 differential equation. Hence, it allowed simulating even with n=100 phosphorylations. Thus we could scale up the simulations from n=4 to n=100 [28].

Transcriptional-translational feedback loops (TTFLs) consisting of phosphorylated clock proteins are known to generate self-sustained rhythms [1, 7]. Therefore, we devised a fusion model that integrates a Goodwin oscillator, without ad hoc Hill coefficient, with distributive multiple random phosphorylations [32, 53, 54].

**Findings:** Interestingly we found that even just the linear phosphorylations could produce required delays. In comparison to linear phosphorylations, nonlinear phosphorylations provided higher Hill coefficients. Amplitudes decreased considerably for higher phosphorylation levels [28].

Using nonlinear models we showed that enzyme sequestration could enhance switch-like behaviour. For higher kinase levels threshold behaviour was observed. Such threshold phenomena might be due to the sequestration of enzymes by different species of phosphorylated proteins. In addition to delayed temporal switches and threshold behaviour, we found interesting nonlinearities of steady states governed by sequestration for the varying kinase production [28].

Using random model we showed that the main properties, i.e. large amplitudes at intermediate phosphorylation levels, long delays, and high Hill coefficients, are robust with respect to large parameter changes [28].

Furthermore, the negative feedback in TTFL is realized by increasing phosphorylation levels. Interestingly, active and inactive species of FRQ protein seem to produce rhythms as observed also in experimental studies [55, 56, 57]. We conclude that delayed switch-like behavior due to slow random multiple phosphorylation appears to be key design principle in circadian rhythm generation.

### 2.1.3 Publication: Multiple random phosphorylations in clock proteins provide long delays and switches

The publication [28] with DOI: https://doi.org/10.1101/2020.06.07.138438 is available at: `http://www.nature.com/articles/s41598-020-79277-z`.

### 2.1.4 Discussion

**Question and findings** In this work we took inspiration from recent experimental observations of multiple phosphorylations of clock proteins [15, 16, 58, 59]. We utilized generic mathematical models to address two principal questions: (i) can long delays be realized with multiple random phosphorylations? and (ii) what is the role of phosphorylations in switch-like behaviour?

Previously, simple models have been used to study the role of one or two phosphorylation sites onto clock related mechanisms [13, 60]. For example, modelling proposed a functional role of a mutated phosphorylation site of mammalian clock protein PER in familial advanced sleep phase syndrome (FASPS) [13]. However, the role of phosphorylation in self-sustained oscillations is not well understood. A *Neurospora* clock like other clocks do rely on robust delays and nonlinearities. Multiple phosphorylations of FRQ became a good candidate in view of the experimentally identified phosphorylation sites. We, therefore, built conceptual models of sequential and distributive phosphorylations. Our simple models revealed that multiple phosphorylations and sequestration mechanisms not only provide delays but also a switch-like dynamics in the *Neurospora* clock.

Notably, in addition to the temporal switches, we also found a steady state switch. Sequestration of enzymes provided a threshold behaviour and an increasing enzyme levels led to the steady state switches. Furthermore, we extended the nonlinear model from 4 sites of FRQ to more realistic random phosphorylation of 100 sites. Our random model highlighted the robust temporal switches with long delays and high amplitudes for intermediate phosphorylation levels. Thus, our simulations suggested that the slow and seemingly random phosphorylations of FRQ regulate the function and stability of FFC complex. This reinstated, consistent with the recent experiments, a delayed negative feedback due to multiple random phosphorylations [52, 59, 61].

**Limitations and proposed extensions of the model** Our generic models, however, were constrained by limited experimental data. Nevertheless, our analysis reflected upon an in vitro fully phosphorylated 100 sites of FRQ in about 48 hours and showed an experimentally observed phosphoswitch in the *Neurospora* clock [15, 17, 62].

In a circadian day, FRQ frequently interacts with CK1a, FRH, WC1 and FWD1 via the corresponding domains [62]. The multiple phosphorylations of 100 sites on FRQ could be spanned over domain specific sites. A critical number of phosphorylated sites on any of these domains may lead to the overall switch in the activity of FRQ [15]. Simulations and experiments may indicate the functional role of the FFC switch in a domain-specific manner.

Interestingly, the binding and unbinding of kinase (free and bound kinase) on FCD (FRQ-CK1a-Domain) might be interlocked to each other. CK1a first makes a stable binding to FCD of unphosphorylated FRQ. Hyperphosphorylations of a FRQ molecule may trigger CK1a unbinding from that moleculae and binding to another

unphosphorylated molecule [62]. This coupling mechanism via a positive feedback from one molecule to another may give rise to cooperativity. Therefore, a switch-like behaviour can also arise due to FRQ and CK1a interaction via FCD.

Additionally, an experiment indicates that CK1a has the potential to hyperphosphorylate FRQ at many sites in priming-independent and seemingly stochastic manner [25]. The overall reaction is slow and temperature-compensated. Recruitment of CK1a to FRQ is required to support this low affinity reaction. Phosphorylation of FRQ by bound CK1a relies on intrinsically disordered parts of FRQ overturning into the catalytic site of the kinase. Consequently, autoinhibited CK1a attached to disordered FRQ accounts for an hourglass with an ability to progressively hyperphosphorylate FRQ in a temperature-compensated manner [15]. Therefore, modelling may indicate an intrinsic temperature compensation mechanism which could be realized by two oppositely temperature dependent reactions: CK1a binding increases with decreasing temperature, whereas the catalytic activity of CK1a decreases.

Furthermore, other experiments suggest that priming dependent phosphorylation of FRQ by CK1a is not required for the clock function but seems to affect clock period. Although FRQ phosphorylation by CK1a is largely priming-independent, several sites in FRQ match the CK1 consensus for priming-dependent phosphorylation. Since CK1a shows a higher affinity towards primed than for unprimed sites, priming-dependent phosphorylation is independent of the recruitment of CK1a to FRQ [25]. In this regard, two modes of phosphorylations by CK1a could be incorporated into the models. Theoretical analysis may suggest an important regulatory and compensatory functions of priming-dependent phosphorylation in the *Neurospora* circadian clock.

## 2.2 Published work 2: a transcriptional inactivation switch allows rhythms in a circadian clock

### 2.2.1 Context

**Debated mechanism of negative feedback:** The basic design principle of transcription-translation feedback loops (TTFL) is evolutionarily conserved across the kingdoms of life [3]. The molecular components are however different from species to species. Indeed, a delayed TTFL with nonlinearities is the key generator of circadian rhythms in *Neurospora* [8, 9]. We above discussed how a long delay and switch-like behaviour could be achieved with multiple phosphorylations of FRQ. However the detailed mechanisms behind the switch-like inhibition of WCC-induced frq transcription are not well understood.

The gene frequency is a negative element of the clock. The FFC complex is necessitated in the autoinhibition of frq transcription. Moreover, the inhibition of WCC by FFC might involve hyperphosphorylation, stoichiometric repression, and sequestration [10, 18]. Therefore, we searched for quantitative details of the delayed negative feedback and conceived of a transcriptional regulation switch.

**Plausible mechanisms for glucose compensation of circadian periods:** Circadian clocks in nature exhibit three main properties: 1) a period of about 24 hours, 2) temperature and glucose compensation, and 3) entrainability by environmental cues. Glucose or nutrient compensation means that the period of circadian clock does not change significantly with fluctuating glucose concentration in cells [9]. The glucose compensation mechanism of *Neurospora* clock is however not properly elucidated.

An additional transcriptional feedback loop via CSP1 was recently suggested for glucose compensation [63, 64]. Our model contained only the core negative feedback loop of FRQ and a positive feedback on WC1 [20]. We therefore focused on finding an in-built mechanism without additional feedbacks. The high glucose is thought to have an increasing effect on transcription and translation rates of FRQ and WC1. Thus, we hypothesized that the corresponding period changes due to increased rates could play a crucial role in compensation.

Interestingly, a moonlighting protein could also offer a glucose compensatory mechanism. The dual role of FRH in *Neurospora* clock is well known. FRH is found to act as an RNA helicase and as a stabilizer of the FFC complex [65, 66]. It is known that a higher number of mRNAs is transcribed under high glucose conditions. FRH is therefore sequestered. This in turn leads to the destabilization of FFC complex [65]. The shorter half life of FRQ therefore lead to a shortening of the period. We therefore conceived that the dual behaviour of FRH could be a potential candidate for glucose compensation.

### 2.2.2 Research question and findings

**Research question:** Given the complexity of the TTFL loop shown in Figure 2.2 and the debated negative feedback mechanisms contained in this network, we asked how a strong negative feedback is realized.

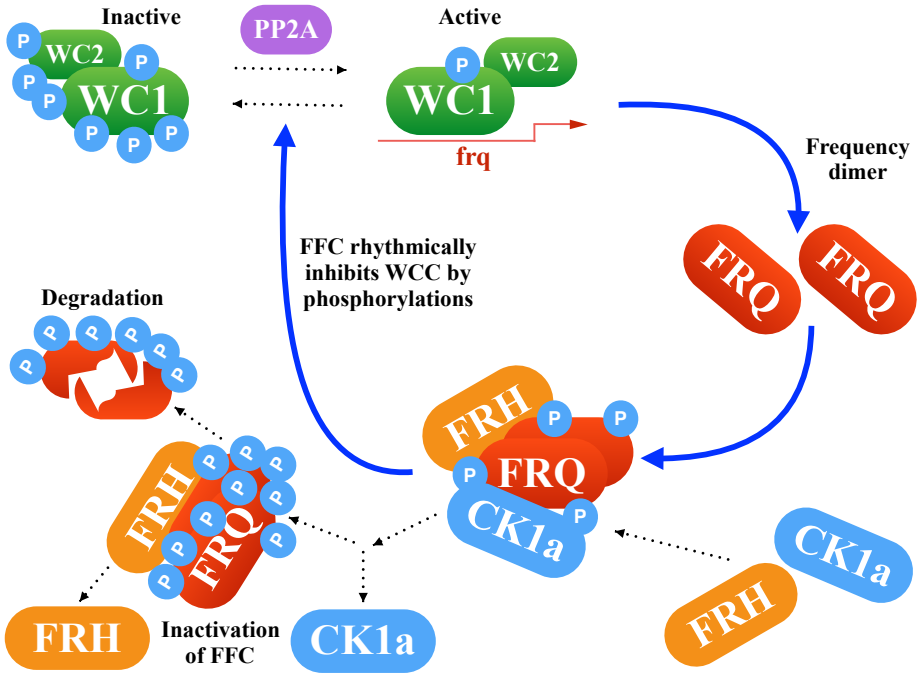


FIGURE 2.2: Core circadian clock network of *Neurospora*. Taken from [20]: The transcription translation feedback loop of FRQ and rhythmic inactivation of the WCC-induced frq transcription. This delayed negative feedback is governed by intermediate processes such as mainly complex formations, phosphorylations, and degradation.

There are experimental data on half-lives of FRQ in its dimer form, complexes with CK1a and FRH, and interaction with WCC. Fig 2.2 reflects a long chain of processes involved in the negative feedback loop of the *Neurospora* clock. The FRQ-dimer is progressively phosphorylated by CK1a and assembles into the FFC complex [9, 42, 43]. We were therefore interested, how interactions between FFC and WCC provide switch-like inhibition of frq trancription needed for negative feedback. While exploring their temporal dynamics, we investigate how self-sustained rhythms arise and how molecular switches favour oscillations.

In addition, we studied the compensations of environmental fluctuations. It has been found experimentally that the period of *Neurospora* clock is maintained almost constant at changing temperatures and glucose concentrations [64, 67, 68]. We therefore explored the mechanisms of glucose compensation.

**Approach:** We extended an ordinary differential equation model of the *Neurospora* core circadian clock [69]. Here, we methodically explored the mechanisms of delayed switch-like inhibition in negative feedback loops and the origin of self-sustained rhythms. Using methods from control analysis we also investigated the mechanisms for glucose compensation.

The processes such as transcription, translation, nuclear import and export, complex formations, post-transcription and post-translation modifications provide sufficient delays in *Neurospora* clock. Experiments show that the half-lives of FRQ protein are in the range of 3-5 hours [9, 42]. We developed a minimal model of 10 ODEs and 26 parameters of a long chain of intermediate processes. We, to this end, simulated

the time series of the model variables.

Oscillator theory suggests that nonlinearities are required to obtain self-sustained rhythms [26, 70]. Our model contained just three nonlinearities: Hill coefficient of only 2 and two other bilinear terms. We therefore replaced the nonlinear terms by their mean (called "clamping") or by a linearized kinetics to verify their relevance [71]. Moreover, we performed comprehensive bifurcation analysis in order to quantify the robustness of the model [72].

There is some evidence that the helicase FRH has a dual role in the *Neurospora* clock. FRH degrades nascent mRNAs and assembles into the FFC complex [65, 66]. Therefore, we explored the role of FRH in glucose compensation mechanisms using sensitivity analysis [35]. We changed the parameters value by $\pm$ 10 percent and calculated the corresponding period changes. Thus, we systematically calculated the control coefficients to quantify period changes with respect to changes in parameters.

**Findings:** Interestingly, we found that our model showed limit cycle oscillations with minimal nonlinearities. We observed sinusoidal oscillations of frq mRNA and protein, although the inhibitory complex FFCn and the transcription factor WC1n showed spike-like waveforms. Interestingly, the FFCnWC1n complex displayed harmonics: two peaks within one circadian cycle [73]. Simulations are consistent with the experimentally observed ultradian rhythms in large-scale transcription profiles in *Neurospora* [74]. In addition, simulations reproduced the experimentally found clock mutants for shorter and longer period (frq1 and frq7), and proteasomal knockout ($\Delta$fwd1) [10, 55, 75].

Notably, bifurcation analysis showed that the self-sustained rhythms with the wildtype (frq+) period of 22.5 hours were maintained in broad ranges of parameters [10]. Surprisingly, the oscillations continued even after clamping or linearizing two of the bilinear terms. This analysis highlighted the limited role of the positive feedback and dimerization to get self-sustained rhythms. Thus, we discovered that the Hill function describing frq transcription turned out to be only essential nonlinearity in our model. In addition, the frq transcriptional motif showed a strong ON/OFF temporal switch. A closer inspection of the levels of FFC, WC1, and FFC-WC1 complex revealed a FFC-assisted inactivation of WC1.

Furthermore, we searched for possible compensatory mechanisms. We, suprisingly, found that the period increase via faster FRQ and WC1 production alone led to the compensation under high glucose levels. Interestingly, an increased transcription due to high glucose, and a slower assembly plus less stabilization of FFC due to sequestration of the helicase FRH could also compensate the period [65]. Notably, the glucose compensation through the dual role of FRH faded away upon the removal of the positive feedback [20]. Thus, we uncovered two plausible in-built mechanisms of glucose compensation.

All in all, we found a switch between active transcription to FFC-aided rapid inactivation. We conclude that a transcriptional inactivation switch enables self-sustained circadian rhythms in *Neurospora* clock. Additionaly, our extensive control analysis revealed two possible mechanisms of glucose compensation.

### 2.2.3 Publication: An inactivation switch enables rhythms in a *Neurospora* clock model

The publication [20] with DOI: https://doi.org/10.3390/ijms20122985 is available at: `https://www.mdpi.com/1422-0067/20/12/2985/htm`.

### 2.2.4 Discussion

**Question and approach** In this publication, we approached the circadian clock network of *Neurospora* by constructing a mathematical model. We analyzed the spatio-temporal interactions of core clock genes and proteins, leading to our main result that "an inactivation switch enables rhythms in a *Neurospora* clock model".

A variety of models have been introduced to study the mechanism of the *Neurospora* circadian clock. They focused on a negative feedback loop alone, interconnected negative and positive feedback loops, or additional feedbacks loop. Our model varies from those of [69, 76, 77] by considering explicitly FRH and CK1a in an interlocking negative and positive feedback loop. It was particularly developed using new experimental data on half-lives of FRQ and known degradation rates of FRQ and WCC [64, 65]. Our model revealed the mechanism of switch-like inhibition in a negative feedback loop. Additionally, we studied the behaviour of the model and observed that bifurcations and major period changes do not take place near the default parameter set. This upholds the robustness of the model. While a temporal switch and co-existence of the attractors were observed, no steady-state switch was found [78].

Interestingly, the FRH is known for stabilizing FRQ independently of its RNA-helicase activity [65, 66]. This dual role of FRH was analyzed in our mathematical model leading to the prediction of glucose compensation mechanisms. Sensitivity analysis predicted that the FRH might act as a metabolic sensor that measures newly synthesized nuclear RNA to indirectly coordinate the circadian clock with metabolism and cell growth. Surprisingly, we found a possible implication of the positive feedback in glucose compensation. When this additional regulation was diminished using clamping, an inherent compensation mechanism was lost.

**Limitations and possible expansions of the model** Our model, however, was not quantitative in all details. Nevertheless, our analysis reproduced some of the key mutants and experimental findings of the *Neurospora* clock [10, 55, 75]. Our semi-quantitative model did not capture all the processes in a negative feedback loop. For example, epigenetic changes may lead to the an active, a refractory and an inactive state of gene expression [14, 79]. Furthermore, a recent experiment shows that the DNA replication is needed for the rhythmic modifications of nucleosome composition at the frq promoter [80]. Thus, a coupling between cell cycle and circadian clock, with DNA methylation and histone modification data, could be modelled. This might refine the mechanisms of the negative feedback at the epigenetic level.

On the other hand, another experiment suggests that the core clock may potentially be regulated by glucose metabolism. CSP1 acts as a repressor of WC1 in a glucose-dependent manner [64]. Moreover, a double negative feedback seems to make the oscillations more robust [63]. Therefore, a double negative feedback consisting of FRQ and CSP1 could be simulated. This will help elucidating a direct role of CSP1, together with FRH, in glucose compensation.

Notably, some of the previous models suggested that the stability of FRQ determines not only the period but also the temperature compensation. The partial loss of temperature compensation in frq7 and frqS513I mutants could be explained by even a simple Goodwin oscillator. This loss was found to be related to an increase in the stability of FRQ which in turn led to a larger period [67, 68]. In other words, a higher

activation energy of FRQ degradation might cause a more temperature-sensitive period. Thus, a mechanism of temperature compensation can be found along the lines of glucose compensation. To do so, model sensitivity analysis can be explored to find a set of parameters oppositely controlling the period at high or low temperature.

Furthermore, higher activation energy of FRQ degradation is found to be correlated to a larger period [67]. An Arrhenius equation can be derived to denote the influence of temperature on any individual process. By incorporating the experimentally found activation energies of FRQ degradation into our model, a temperature compensation mechanism can be studied [67].

# Chapter 3

# General discussion and outlook

## 3.1 The larger perspective: Conclusions and outlook

Most life on earth is directly or indirectly exposed to and affected by the 24 hour day and night cycle. Organisms ranging from cyanobacteria up to mammals have evolved time keeping mechanisms, popularily known as circadian clocks [3]. These molecular clocks enable organisms to anticipate these reoccurring changes and appropriately synchronize their metabolism, physiology and behavior. Self-sustained oscillations persist without environmental signals. Moreover, circadian period is compensated against varying zeitgebers such as light, temperature and nutrients. Thus, circadian clocks are evolutionary universal systems that orchestrate everyday expression rhythms of a substantial number of genes [8, 9, 10].

Technological advances in molecular biology have led to the discovery of new connections between genes and proteins, unfolding a picture of the complexity of the circadian design of biological systems. Such design consists of feedforward and feedback networks [33]. Circadian clocks fundamentally produce rhythmic expression, modification and degradation of clock proteins. Computational and theoretical methods have demonstrated a useful tool for examining and unraveling the spatio-temporal dynamics of involved regulations [29].

However, the mechanisms behind the regulation of feedback loops, circadian rhythms generation and compensations are not well understood. Therefore, the overarching aim of this thesis is to identify and characterize key mechanisms involved in circadian timekeeping. Mathematical theory projects that long delays and nonlinearities are required to generate 24 hour oscillations [29, 81]. Nonlinearities are often associated with molecular switches. These molecular switches are involved in generating or tuning the delays necessary for the generation of 24 hour rhythms. In the two papers we developed mathematical models and extensively employed them to reveal the origin of delays and negative feedback regulations in the *Neurospora* circadian core clock [20, 28]. This strategy empowered us to understand the underlying mechanisms of rhythms generation and nutrient compensation.

In the first publication we attempted to understand the generation of long delays and switch-like behaviour in circadian rhythms [28]. The generic kinetic models used in this study had been carefully conceptualized in agreement with experimental observations. Linear and nonlinear models reflect the multiple random phosphorylations of core clock protein which constitute a processive and distributive mechanism of phosphorylations by kinases. Therefore, models were conceptualized to explore the source of delays and functional inactiavtion of core clock protein upon hyperphosphorylation. We found that extended reaction chains and slow degradation are involved in long delays. Moreover, sequestration led to ultrasensitive temporal switches. A phospho-switch is effectuated by multiple phosphorylation of

clock protein. Interestingly, we found a new feature in random models where amplitudes of intermediate phospho-states accumulated high. Notably, such an increase was due to a combinatorial outburst of molecule types associated with intermediate phosphorylation numbers. We deduced that a delayed switch due to multiple phosphorylations is fundamental to the circadian clock.

In the second publication we attempted to explore the mechanisms of negative feedback and glucose compensation using a new model [20]. On a molecular level, a core circadian transcription factor activates the expression of a large set of genes, among them one or more negative elements (inhibitors), which accumulate and inactivate the transcription factor. In the course of a day, the inhibitory protein is transcribed, translated, functionally modified by phosphorylations and other posttranslational modifications and eventually degraded. This releases repression and closes the circadian loop by reactivation of the transcription factor. Apart from predicting the behaviour of such systems, tracking down the essential regulations which form the building blocks that underlie their behaviour has been a difficult challenge. Therefore, we exploited our model to explore the transcriptional regulation. Indeed, we found that an inactivation of the inhibitory complex followed by the resumption of inhibitor's transcription by activator served as a switching mechanism for negative feedback. Moreover, antagonistic regulations on period by various processes suggested us to perform an extensive sensitivity analysis. The analysis indicated two inbuilt compensatory mechanisms for glucose intake. In summary, we developed a robust model by extending the negative loop via inhibitory complexes and by strengthening the positive feedback. An inactivation switch reproduced simulations of several clock mutants. Intrinsic glucose compensation mechanisms was a key feature of our *Neurospora* clock model.

An apprehension of multiple phosphorylations may guide theoretical and experimental exploration of other posttranslational modifications such as SUMOylation and ubiquitination of the core clock proteins [15]. A reverse engineering of the circadian clock model can be applied to find mechanisms of glucose and/or temperature compensation in other organisms.

We used *Neurospora crassa*, a well established chrono-genetics model organism, to ask fundamental physiological questions. However, several applied research questions on clock's implications into cancer, sleep and metabolic disorders are of general interest [4, 5, 6]. *Neurospora* although has about 28 cell types and multiple nuclei, circadian outputs are mainly studied from 2 cell types- filamentous hyphae and conidiation formation [3].

Remarkably, high-throughput approaches allow to explore the core clock at a systems level. Clock's topical connections ranges from nervous, immune to reproductive systems. The existence of the clocks in the living organisms could largely be for reproductive fitness, host-pathogen interactions and optimizing the energy production [3, 82, 83]. In the course of evolution, organisms are expected to adapt to the varying circumstances. Thus, related ecological and evolutionary questions can be addressed using *Neurospora*.

Our modelling approach was in tune with the limited experimental data. High-resolution single cell technology provides heterogeneous temporal informations. Stochastic model simulations can be employed to study those data [84].

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